The present invention is directed to methods of identifying variant recombinases that mediate recombination at variant recombination sites and producing site-specific recombination of DNA. The methods for identification of variant recombinases comprise, *inter alia*, a) bringing into contact a mutant recombinase, a first nucleic acid sequence comprising a first reporter gene and first and second recombination sites, *wherein the first and second recombination sites are variant recombination sites,* and a second nucleic acid sequence comprising a second reporter gene and third and fourth recombination sites, wherein the third and fourth recombination sites can be recombined by a non-mutant recombinase. It is important to note that the first and second sites are, ***both***, variant recombinant sites.

Rejection Under 35 U.S.C. § 112, first paragraph

Claims 1-47 and 49 were rejected under 35 U.S.C. § 112, first paragraph, as not being enabled. Applicants respectfully traverse this rejection.

If the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. It should be noted that the Applicants are claiming a method of identifying variant recombinases based upon reporter activity. The simplicity and ability of Cre to function *in yeast and mammalian **cells*** has allowed Cre assisted site-directed recombination to become an important tool for efficient, specific, and conditional manipulations of eukaryotic genomes (see lines 28-7, bridging pages 2 and 3 of the specification). The Cre recombinase is derived from the ***bacteriophage*** (i.e. viruses that have a specific affinity for and infect bacteria) P1 (see page 1, lines 14-15). Cre mutants have been studied extensively *in vivo*, assessing a wide

variety of phenotypes based upon the ability of mutant Cre protein to excise a *lacZ* gene located between two loxP sites (see Wierzbicki *et al.*, 1987 – as disclosed in the IDS filed on February 12, 2001).

Claims 1-23 are directed to identifying variant recombinases based upon reporter gene/product activity. The claimed method is predicated on a system providing the proper "environment" for Cre activity. Based upon the teachings of the specification, one of ordinary skill in the art could easily set up the proper "control" assays to ensure that the system is amenable to mutant and wild type Cre activity, without undue experimentation.

Claim 24, and claims dependent thereon, are directed to a method for producing site-specific recombination of DNA using the variant recombinase identified by the method of claim 1.

Rejections Under 35 U.S.C. § 102

Claims 1-6 and 21 were rejected under 35 U.S.C. § 102(b) as being anticipated by *Cell*, Vol. 20, pp. 721-729 (1980) by Miller *et al.* ("Miller"). Claims 1-6 and 21 were rejected under 35 U.S.C. § 102(b) as being anticipated by *J. Biol. Chem.*, pp. 455-463, (1993), by Serre *et al.* ("Serre"). Claims 1-6 and 21 were rejected under 35 U.S.C. § 102(b) as being anticipated by *J. Mol. Biol.* (1990), 216, pp. 633-643, by Ackroyd *et al.* ("Ackroyd"). Applicants respectfully traverse these rejections.

The claims are directed to, in part, methods for identifying a mutant recombinase, where the mutant recombinase is tested for activity on wild type and mutant recombination sites. It should be noted that the recombination events required to characterize the mutant recombinase

recombine two variant sites and/or two wild type sites. The claims are not directed to recombining a wild type site and a mutant site.

Miller

Miller teaches the properties of bacteria lacking a host protein required for lambda integration. "These strains are defective are defective for lambda site-specific recombination due to mutations, in the *E. coli himA* gene" (see page 721, 2nd column). Miller also teaches the isolation and characterization of an int protein that is active in a variety of mutants normally defective in lambda site-specific recombination. Lambda-promoted gal expression is dependent upon the lamba N protein. It should be noted that the secondary sites (i.e. those suggested by the Examiner as being "variant") are the same as those used by lambda-int$^+$. Therefore, by definition, the secondary sites of Miller are not variant recombination sites that ***are not*** recognized by non-mutant recombinase (see page 4, lines 5-8, of the present application, wherein "the constructs contain variant recombination sites that are not recognized by non-mutant recombinase but will undergo recombination in the presence of a mutant recombinase with altered specificity"). With regard to the non-paradigm att sites, the data supplied in column 2, of page 725, is clearly directed to recombination between one variant site and one non-variant site (i.e. "One variant carries the att24 mutation in attL and the other in attR.....17% for lambda-attL24-attR and to 11% for lambda-attL-attR24."). However, the claims as pending are directed to, in part, "a first nucleic acid sequence comprising a first reporter gene and first and second recombination sites, *wherein the first* ***and*** *second recombination sites* ***are variant recombination***

sites" (emphasis added). In all of the cases presented in Miller, recombination proceeds between a wild type site and a variant site.

Serre

Serre teaches the reactions of a Flp half-site with wild-type Flp and two Flp variants, Flp (Y343F) and Flp (H305L). As stated on page 455, second column, an active site mutant of Flp, Flp (Y343F), mediates strand transfer with a half recombination site. "The efficiency of this reaction is low, but the product of the reaction ***is identical to that yielded by wild type Flp***" (see second column, page 455 – emphasis added). Therefore, the half recombination site cannot be one that is ***not*** recognized by a non-mutant recombinase. Moreover, the DNA recognition element(s) in the Serre sites are not variant.

Ackroyd

Ackroyd teaches the ability of Tn*21* resolvase mutants to carry out site-specific recombination between res sites from either Tn*21* or Tn*3*. Table 1 illustrates the site-specific recombination studies using Tn*21* resolvase mutants. With the exception of the E173L mutant, each of the mutants gave way to virtually the same levels of recombination with regard to Tn*21* res (pAA3) (it should be noted that the Tn*3* resolvase cannot be compared to Tn*21*, for the purposes of the pending claimed methods, because Tn*3* is a different resolvase - it is a wild type Tn*3* resolvase and possesses a distinct specificity for DNA recognition). The transformants with either pEAK6 (wild type Tn*21*) or any of its mutants all yielded approximately the same number of colonies in both the presence and absence of tetracycline (see Table 1 and page 638, second column). Again, as with the above-identified references, the data presented in Ackroyd clearly

teaches variant resolvases that recognize DNA ***not unlike their wild-type counterpart(s)***. Variant sites, as defined for the presently pending claims, ***are not*** recognized by non-mutant recombinases (see page 4, lines 5-8). The ability of the Tn21 resolvase and each of the mutants to provide 100% recombination of Tn21 res (E173L = 7%) is a direct contradiction of what the present application teaches.

Rejection Under 35 U.S.C. § 103

Claims 1-30 and 32-49 were rejected under 35 U.S.C. § 103(a) as being unpatentable over by *Cell*, Vol. 20, pp. 721-729 (1980) by Miller *et al.* ("Miller"), or *J. Biol. Chem.*, pp. 455-463, (1993), or Serre *et al.* ("Serre"), *J. Mol. Biol.* (1990), 216, pp. 633-643, by Ackroyd *et al.* ("Ackroyd"), in view of U.S. Patent No. 5,677,177 to Wahl et al. ("Wahl"). Applicants respectfully traverse these rejections.

As stated in the foregoing sections, none of the Miller, Serre, and Ackroyd references teach the identification of a mutant recombinase, wherein the mutant recombinase recombines first and second sites that are *both* variant. Wahl fails to teach a variant recombinase, or any alteration of substrate specificity. Therefore, even in combination, there is nothing that would lead one skilled in the art to the claimed method.

Summary

There is no teaching in the prior art, individually or in combination, of a variant recombinase with altered specificity (i.e. recognizing sequences that are ***not*** recognized by non-mutant recombinases). Furthermore, there is no teaching of assessing the recombination of two sites, wherein the two sites are variant. Applicants have enclosed a copy of a reference by

Yoziyanov *et al.* (Nucleic Acids Res., 2002, Vol. 30, No.7, pages 1656-1663), published after the filing date of this application, which extols the novelty of using a double recombination reporter strategy, as claimed, providing independent third party evidence of the novelty and non-obviousness of the claimed method.

Allowance of claims 1-49 is respectfully solicited.

Respectfully submitted,

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Certificate of Mailing Under 37 C.F.R. § 1.8(a)

I hereby certify that this paper, along with any paper referred to as being attached or enclosed, is being deposited with the United States Postal Service on the date shown below with sufficient postage as first-class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Aisha Wyatt

Date: December 27, 2002

MARKED UP VERSION OF AMENDMENTS PURSUANT TO 37 C.F.R. § 1.121

Clean Version of Pending Claims
Pursuant to 37 C.F.R. § 1.121(c)(1)(ii)

1. A method of identifying variant recombinases that mediate recombination at variant recombination sites, the method comprising,

(a) bringing into contact

a mutant recombinase,

a first nucleic acid sequence comprising a first reporter gene and first and second recombination sites, wherein the first and second recombination sites are variant recombination sites, and

a second nucleic acid sequence comprising a second reporter gene and third and fourth recombination sites, wherein the third and fourth recombination sites can be recombined by a non-mutant recombinase,

(b) determining if recombination occurs between the first and second recombination sites, and determining if recombination occurs between the third and fourth recombination sites,

wherein recombination between the first and second recombination sites indicates that the mutant recombinase is a variant recombinase that mediates recombination at variant recombination sites,

wherein recombination between the third and fourth recombination sites indicates that the mutant recombinase retains the ability to mediate recombination at non-variant recombination sites.

2. The method of claim 1 wherein the recombination sites comprise recognition sequences and compatibility sequences,

wherein the recognition sequences of the first and second recombination sites differ from the recognition sequences of the third and fourth recombination sites,

wherein the compatibility sequences of the first and second recombination sites are sufficiently similar to allow recombination between the first and second recombination sites, and wherein the compatibility sequences of the third and fourth recombination sites are sufficiently similar to allow recombination between the third and fourth recombination sites, and

wherein the compatibility sequences of the first and second recombination sites differ from the compatibility sequences of the third and fourth recombination sites such that neither the first nor the second recombination site can be recombined with either the third or the fourth recombination site.

3. (Amended) The method of claim 1 wherein recombination frequency between the first and second recombination sites mediated by a non-mutant recombinase is significantly reduced.

4. The method of claim 1 or 2 wherein the first and second recombination sites have identical sequences, and wherein the third and fourth recombination sites have identical sequences.

5. The method of claim 1 wherein recombination between the first and second recombination sites alters the expression of the first reporter gene, wherein recombination

between the first and second recombination sites is determined by determining if expression of the first reporter gene is altered, and

wherein recombination between the third and fourth recombination sites alters the expression of the second reporter gene, wherein recombination between the third and fourth recombination sites is determined by determining if expression of the second reporter gene is altered.

6. The method of claim 5 wherein recombination between the first and second recombination sites allows the first reporter gene to be expressed.

7. The method of claim 6 wherein the first nucleic acid sequence further comprises a spacer sequence flanked by the first and second recombination sites, wherein the spacer sequence interrupts the first reporter gene such that the first reporter gene is not expressed, wherein recombination of the first and second recombination sites excises the spacer sequence which allows the first reporter gene to be expressed.

8. The method of claim 6 wherein a portion of the first reporter gene is inverted, wherein the inverted portion of the first reporter gene is flanked by the first and second recombination sites, wherein recombination of the first and second recombination sites inverts the inverted portion of the first reporter gene which allows the first reporter gene to be expressed.

9. The method of claim 5 wherein recombination between the first and second recombination sites prevents expression of the first reporter gene.

10. The method of claim 9 wherein the first reporter gene is flanked by the first and second recombination sites, wherein recombination of the first and second recombination sites excises the first reporter gene which prevents expression of the first reporter gene.

11. The method of claim 9 wherein a portion of the first reporter gene is flanked by the first and second recombination sites, wherein recombination of the first and second recombination sites inverts the flanked portion of the first reporter gene which prevents expression of the first reporter gene.

12. The method of claim 5 wherein recombination between the third and fourth recombination sites allows the second reporter gene to be expressed.

13. The method of claim 12 wherein the second nucleic acid sequence further comprises a spacer sequence flanked by the third and fourth recombination sites, wherein the spacer sequence interrupts the second reporter gene such that the second reporter gene is not expressed, wherein recombination of the third and fourth recombination sites excises the spacer sequence which allows the second reporter gene to be expressed.

14. The method of claim 13 wherein the spacer sequence interrupts the second reporter gene such that the second reporter gene is not transcribed.

15. The method of claim 13 wherein the second reporter gene encodes a protein, wherein the spacer sequence interrupts the second reporter gene such that the protein encoded by the second reporter gene is not translated.

16. The method of claim 13 wherein the spacer sequence interrupts the second reporter gene such that the second reporter gene produces an inactive expression product.

17. The method of claim 12 wherein a portion of the second reporter gene is inverted, wherein the inverted portion of the second reporter gene is flanked by the third and fourth recombination sites, wherein recombination of the third and fourth recombination sites inverts the inverted portion of the second reporter gene which allows the second reporter gene to be expressed.

18. The method of claim 5 wherein recombination between the third and fourth recombination sites prevents expression of the second reporter gene to be expressed.

19. The method of claim 18 wherein the second reporter gene is flanked by the third and fourth recombination sites, wherein recombination of the third and fourth recombination sites excises the second reporter gene which prevents expression of the second reporter gene.

20. The method of claim 18 wherein a portion of the second reporter gene is flanked by the third and fourth recombination sites, wherein recombination of the third and fourth recombination sites inverts the flanked portion of the second reporter gene which prevents expression of the second reporter gene.

21. The method of claim 1 wherein the first nucleic acid sequence is a first nucleic acid construct and the second nucleic acid sequence is on a second nucleic acid construct.

22. The method of claim 21 wherein the first nucleic acid construct is an extrachromosomal vector and the second nucleic acid construct is in the genome of a host cell.

23. The method of claim 1 wherein the first and second nucleic acid constructs are on the same nucleic acid construct.

24. (Amended) A method for producing site-specific recombination of DNA, comprising,

contacting a variant recombinase identified by the method of claim 1 with third and fourth DNA sequences,

wherein the third DNA sequence comprises a fifth recombination site and the fourth DNA sequence comprises a sixth recombination site,

wherein the variant recombinase mediates recombination between the fifth and sixth recombination sites thereby producing the site specific recombination.

25. (Amended) The method of claim 24 wherein the fifth recombination site, the sixth recombination site, or both, are variant recombination sites.

26. (Amended) The method of claim 24, wherein the third and fourth DNA sequences are connected by a pre-selected DNA segment.

27. (Amended) The method of claim 26, wherein the fifth and sixth recombination sites have the same orientation and the site-specific recombination of DNA is a deletion of the pre-selected DNA segment.

28. The method of claim 27, wherein the pre-selected DNA segment is a gene for a structural protein, an enzyme, or a regulatory molecule.

29. (Amended) The method of claim 27 further comprising contacting the variant recombinase with a fifth DNA sequence comprising a seventh recombination site, wherein the fourth and fifth DNA sequences are connected by a second pre-selected DNA segment.

30. (Amended) The method of claim 29 wherein the fifth recombination site is a variant recombination site recognized by the variant recombinase and not by wild type recombinase, and wherein the sixth and seventh recombination sites are recombination sites recognized by wild type recombinase and by the variant recombinase.

31. (Amended) The method of claim 30 further comprising, prior to contacting the variant recombinase with the fifth, sixth, and seventh recombination sites, contacting the recombination sites with wild type recombinase, thereby producing site specific recombination between the sixth and seventh recombination sites resulting in a deletion of the second pre-selected DNA segment.

32. The method of claim 29, wherein the second pre-selected DNA segment is a gene for a structural protein, an enzyme, or a regulatory molecule.

33. (Amended) The method of claim 26, wherein the fifth and sixth recombination sites have opposite orientations and the site-specific recombination is an inversion of the nucleotide sequence of the pre-selected DNA segment.

34. (Amended) The method of claim 33, wherein the fifth and sixth recombination sites are variant recombination sites recognized by the variant recombinase.

35. The method of claim 33, wherein the pre-selected DNA segment is a gene for a structural protein, an enzyme, or a regulatory molecule.

36. (Amended) The method of claim 24, wherein the fourth and fifth DNA sequences are introduced into two different DNA molecules and the site-specific recombination is a reciprocal exchange of DNA segments proximate to the recombination sites.

37. (Amended) The method of claim 36, wherein the fifth and sixth recombination sites are variant recombination sites recognized by the variant recombinase.

38. (Amended) The method of claim 24 wherein the fourth DNA sequence includes a label, wherein recombination between the fifth and sixth recombination sites associates the label with the third DNA sequence.

39. (Amended) The method of claim 38 wherein the third DNA sequence is a large circular DNA molecule.

40. The method of claim 24 wherein recombination occurs in a cell.

41. (Amended) The method of claim 40 wherein the variant recombinase is contacted with the third and fourth DNA sequences by introducing into the cell a sixth DNA sequence comprising DNA encoding the variant recombinase.

42. (Amended) The method of claim 41, wherein the sixth DNA sequence further comprises a regulatory nucleotide sequence and expression of the variant recombinase is produced by activating the regulatory nucleotide sequence.

43. The method of claim 40, wherein the cell is a eukaryotic cell, a mammalian cell, a yeast cell, a fungal cell, a prokaryotic cell, a bacterial cell, an archae bacterial cell, or a cell in a multicellular organism.

44. The method of claim 43 wherein the multicellular organism is a plant, an animal, or a mammal.

45. (Amended) The method of claim 40, wherein the third and fourth DNA sequences are connected by a pre-selected DNA segment, wherein the first and second

recombination sites have the same orientation and the site-specific recombination of DNA is a deletion of the pre-selected DNA segment.

46. The method of claim 45, wherein the cell is in a multicellular organism.

47. The method of claim 45, wherein the pre-selected segment is an undesired marker or trait gene.

48. The method of claim 24, wherein the variant recombinase is contacted with the recombination sites *in vitro*.

49. The method of claim 48, wherein the method further comprises introducing the recombined DNA into a cell.

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